Immediate protection of swine from foot-and-mouth disease: a combination of adenoviruses expressing interferon alpha and a foot-and-mouth disease virus subunit vaccine

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Abstract

We have previously shown that swine inoculated with recombinant, replication-defective human adenovirus type 5 containing the porcine interferon alpha gene (Ad5-pIFNα) are completely protected when challenged 1 day later with virulent foot-and-mouth disease virus (FMDV). In the current study, we examined the duration of protection afforded swine by Ad5-pIFNα and the ability of a combination of Ad5-pIFNα and a FMDV subunit vaccine delivered by Ad5-A24 (an Ad5 vector containing the capsid coding region of FMDV serotype A24 Cruzeiro and the 3C proteinase coding region of FMDV serotype A12) to induce immediate as well as long-lasting protection against homologous FMDV challenge. Groups of swine were inoculated with Ad5-pIFNα and challenged with virulent FMDV A24 1, 3, 5, and 7 days postinoculation (dpi) or 1 day preinoculation. All animals challenged 1 and 3 dpi were completely protected from disease. The animals in the remaining groups had either no clinical signs of disease or clinical signs were delayed and less severe compared to the control group. Swine inoculated with a combination of Ad5-pIFNα and Ad5-A24 and challenged 5 dpi were all completely protected from disease and developed a significant FMDV-specific neutralizing antibody response.
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1. Introduction

Foot-and-mouth disease (FMD) is a highly contagious vesicular disease of cloven-hoofed animals that rapidly replicates and spreads [1]. The disease is caused by a single-stranded, positive-sense RNA virus that is the prototype member of the *Aphthovirus* genus, *Picornaviridae* [2]. Outbreaks of FMD can have severe economic and social consequences as demonstrated by disease incursions in Taiwan in 1997 and in the United Kingdom (UK) in 2001 [3–5]. Both outbreaks resulted in billions of dollars (US) in direct and indirect costs and the slaughter of millions of animals [4,5].

Because FMD is highly infectious, disease-free countries have introduced trade barriers that prohibit importation of animals or animal products from countries that have the disease. Current measures to contain an FMD outbreak include control of animal movement, slaughter of infected and in-contact animals, disinfection, and vaccination. Previously disease-free countries, however, hesitate to use vaccination to eliminate FMD, because the current vaccine, an inactivated preparation of concentrated cell culture derived virus that contains varying degrees of contaminating viral nonstructural (NS) proteins, often induces an antibody response against the NS proteins that can make it difficult to distinguish vaccinated from infected or convalescent animals using currently approved diagnostic tests [6–8]. In addition, vaccinated animals can become long-term carriers following contact with foot-and-mouth disease virus (FMDV) [9,10].

The Office International des Epizooties (OIE: world organization for animal health) is responsible for setting internationally agreed standards relating to animal health. Member nations are required to immediately report the occurrence of FMD and conditions under which a country can be considered free of FMD, and thus resume trade in animals and animal products, have been specified. Currently, countries that slaughter FMD infected animals to control an

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outbreak or vaccinate and destroy these animals can resume FMD-free status more quickly than countries that vaccinate all susceptible animals in a region but do not slaughter them [11]. Thus, the economic benefits of a rapid return to disease-free status have often had a negative impact on the option of using the current vaccine as part of a control strategy. However, as was demonstrated in the UK outbreak, the slaughter of millions of animals raises significant problems, that can affect many aspects of the economic and social life of a country in addition to the animal industry.

We have attempted to develop a disease control strategy that addresses both the concerns of FMD-free countries with the current vaccine and the ability of FMD to rapidly cause disease. Our approach, a combination strategy, includes an FMDV subunit vaccine [7,12] and a prophylactic antiviral treatment [13].

We have produced an FMD vaccine that overcomes many of the limitations of the current inactivated vaccine and yet still induces a protective response after only one inoculation [7]. Our FMD subunit vaccine contains only the capsid and 3C proteinase coding regions of the viral genome, an empty viral capsid construct, and is delivered via a replication-defective human adenovirus type 5 vector (Ad5-FMDV) [12,14]. This vaccine has a number of advantages over the current inactivated virus vaccine including: (1) the absence of infectious FMDV obviating the need for vaccine production in high containment facilities; (2) the absence of the coding regions for a number of NS proteins allowing one to distinguish vaccinated from infected or convalescent animals using a very sensitive diagnostic assay containing the highly immunogenic viral NS protein, $3D^{pol}$; (3) the faithful replication of DNA avoiding the problem of selection of antigenic variants during cell culture passage of Ad5-FMDV as occurs during growth of FMDV for vaccine production. Utilizing this vaccine, we have successfully protected swine challenged 7 days after a single inoculation [7].

An additional concern with the current inactivated virus vaccine as well as with the recombinant Ad5-FMDV vaccine is their inability to induce rapid protection. Both vaccines require approximately 7 days to induce protection in susceptible animals, a time frame that can allow an outbreak to quickly amplify and spread. To address this limitation, we have identified an antiviral compound that can provide rapid protection and is effective against all viral serotypes. Alpha/beta interferon (IFNα/β) is one of the first lines of host cell defense against viral infection [15,16] and we and others have demonstrated that FMDV is highly sensitive to its action [13,17–20]. We recently constructed an Ad5 virus containing porcine IFNα (Ad5-pIFNα) and demonstrated that administration of this virus can protect swine from disease as well as virus replication when challenged with virulent FMDV 1 day later [13].

In the current study, we examined both the duration of protection induced by Ad5-pIFNα inoculation alone and the ability of a combination of Ad5-pIFNα and Ad5-A24

(an Ad5 vector containing the capsid coding region of FMDV serotype A24 Cruzeiro and the 3C proteinase coding region of serotype A12), to afford rapid as well as long-lasting protection. This strategy may enable countries to avoid large-scale slaughter of animals and the subsequent environmental and social concerns that arise.

2. Materials and methods

2.1. Cells and viruses

Human 293 cells were used to generate and grow recombinant Ad5 viruses and determine virus titer [7,21]. In this study, we used recombinant viruses Ad5-pIFNα [13], Ad5-A24 [7], and Ad5-VSVG [22]. The latter two viruses contain the capsid coding region of FMDV serotype A24 Cruzeiro and 3C proteinase coding region of FMDV serotype A12 [7] and the glycoprotein gene of vesicular stomatitis virus-NJ, respectively [22]. Baby hamster kidney (BHK-21) cells (clone 13) were used to measure FMDV titers in plaque assays. Antiviral activity of plasma from inoculated animals was assayed in IBRS2 (swine kidney) cells by a plaque-reduction assay [18]. FMDV serotype A24 (strain Cruzeiro, Brazil, 1955 provided by A. Tanuri from the University of Rio de Janeiro) was amplified by infection of a pig and virus was isolated from vesicular lesions. The 50% bovine infectious dose (BID_{50}) was determined by standard protocols [23].

2.2. Animal experiments

All animal experiments were performed in disease secure isolation facilities at the Plum Island Animal Disease Center following a protocol approved by the Institutional Animal Use and Care Committee. In the first experiment, eighteen Yorkshire gilts, approximately 35–40 lbs, were divided into six groups containing three animals per group and each group was housed in a separate room. All animals were inoculated intramuscularly (IM). Group 1 was inoculated with 10^9 pfu Ad5-VSVG, groups 2–6 were inoculated with 10^9 pfu Ad5-pIFNα (Table 1). The animals were monitored clinically for adverse effects to Ad5-pIFNα administration including fever and lethargy, and for antiviral activity in the plasma. We also used an ELISA to detect the presence of pIFNα in the plasma (see below). The animals in the above groups were challenged with FMDV 7 days postinoculation (dpi) (groups 1 and 2), 5 dpi (group 3), 3 dpi (group 4), and 1 dpi (group 5). The animals in group 6 were challenged with FMDV 1 day prior to inoculation with 10^9 pfu Ad5-pIFNα. All groups were challenged with 3×10^5 pfu swine-derived FMDV serotype A24 (10^5 BID_{50}) in the heel bulb of both rear feet [23,24]. This route of challenge in swine is one recommended by OIE and used previously by us and many other investigators [13,22,24–27]. The animals were monitored for 2 weeks after challenge. Rectal

Table 1
Duration of protection

Group[a]	Challenge[b] (dpi)	IFN[c]	Viremia[d]/dpc[e]	Clinical score[f]/dpc[g]	PRN$_{70}$[h]	NS Ab[i]
1-Control	7					
61-6		0	3.1×10^7/1	17/2	ND[j]	ND[k]
66-11		0	2.3×10^6/1	16/2	8000	+
69-8		0	3.0×10^5/1	14/2	8[l]	ND
2-IFN	7					
70-4		16063	2.5×10^3/2	8/4	8000	+
70-5		17925	1.9×10^3/2	10/5	800[m]	ND
70-6		17000	1.1×10^3/2	12/4	4000	+
3-IFN	5					
70-7		13350	0/7	11/9	6400	+
70-8		21075	0/0	0/0	80	−
71-4		24150	0/7	13/8	6400	+
4-IFN	3					
337-3		13766	0/0	0/0	160	−
338-6		11829	0/0	0/0	128	−
342-6		11354	0/0	0/0	32	−
5-IFN	1					
333-4		5685	0/0	0/0	32	−
335-11		15979	0/0	0/0	64	−
335-9		15748	0/0	0/0	64	−
6-IFN	−1[n]					
81-6		15846	7.0×10^1/1	0/0	2000[o]	ND
81-10		0	2.5×10^5/1	14/2	8000	+
308-9		12506	7.3×10^4/1	10/2	4000	+

[a] Inoculated IM with 10^9 pfu/animal Ad5-VSVG (group 1) or 10^9 pfu/animal Ad5-pIFNα (groups 2–6).

[b] Challenged at indicated day post- or preinoculation with 3×10^5 BID$_{50}$ FMDV A24.

[c] ELISA, pg/ml at 1 dpi.

[d] Viremia 2 dpc determined by plaque assay.

[e] Day postchallenge viremia first detected.

[f] Number of digits and snout with vesicles.

[g] Day postchallenge vesicles first detected.

[h] Neutralizing antibody titer at 14 dpc, or at last bleed before animal died.

[i] Antibodies (14 dpc) against NS proteins detected by RIP.

[j] PRN$_{70}$ not done, 61-6 died 4 dpc.

[k] NS Ab assay not done.

[l] 69-8 died 7 dpc.

[m] 70-5 died 11 dpc.

[n] Day preinoculation.

[o] 81-6 died 8 dpc.

temperatures, lesion data, and physical condition of the animals were determined daily and blood and serum samples were collected as indicated below. Lesion score of the animals were determined at 14 days postchallenge (dpc) by the number of digits plus snout with vesicles.

In the second experiment, 12 Yorkshire gilts, approximately 35–40 lbs, were divided into four groups containing three animals per group and housed in separate rooms (Table 2). The animals in group 1 were inoculated IM with 10^9 Ad5-VSVG, in group 2 with 5×10^9 pfu Ad5-A24, group 3 with 10^9 pfu Ad5-pIFNα, and group 4 with 10^9 pfu Ad5-pIFNα and 5×10^9 pfu Ad5-A24. Five days postinoculation all groups were challenged as described above. The animals were monitored for 3 weeks after challenge and blood, serum, and nasal swabs were collected as indicated.

2.3. Serology and virus titration

Sera samples were collected at 0, 4, 7 and 14 dpc for experiment 1 and 0, 4, 9, 14, and 21 dpc for experiment 2, heated at 56 °C for 30 min, and aliquots stored at −70 °C. Sera were tested for the presence of neutralizing antibodies against FMDV in a plaque-reduction neutralization (PRN) assay [28]. Neutralizing titers were reported as the serum dilution yielding a 70% reduction in the number of plaques (PRN$_{70}$).

Heparinised blood was collected on the day of challenge (0 dpc), daily for the first 7 dpc, and as indicated thereafter in each experiment, and aliquots were frozen at −70 °C. Viremia was determined by a standard plaque assay in BHK-21 cells. Plasma was obtained by centrifugation of heparinised blood at 2500 rpm for 10 min and examined for

Table 2
Co-administration of Ad5-pIFNα and Ad5-A24

Group[a]	IFN[b]	Viremia[c]/dpc[d]	Clinical score[e]/dpc[f]	PRN$_{70}$[g] (0 dpc)	PRN$_{70}$[h] (14 dpc)	NS Ab[i]
1-Control						
9987	52	2.0×10^5/1	14/2	0	32000[i]	+
9988	1867	1.0×10^3/1	13/3	0	16000	+
9989	0	6.1×10^4/1	14/2	0	16000	+
2-A24						
9981	0	0/0	2/3	32	8000	+
9982	395	0/0	6/3	32	8000	+
9983	0	0/3	9/3	64	8000	+
3-IFN						
9975	13718	0/0	0/0	32	64	−
9976	15431	0/0	0/0	0	32	−
9977	16059	0/0	0/0	0	64	−
4-IFN + A24						
9972	17390	0/0	0/0	32	200	−
9973	18631	0/0	0/0	16	400	−
9974	17654	0/0	0/0	32	400	−

[a] Inoculated IM with 10^9 pfu/animal Ad5-VSVG (group 1), 5×10^9 pfu/animal Ad5-A24 (group 2), 10^9 pfu/animal Ad5-pIFNα (group 3), and 10^9 pfu/animal Ad5-pIFNα + 5×10^9 pfu/animal Ad5-A24 (group 4) and challenged 5 days later with 3×10^5 BID$_{50}$ FMDV A24.

[b] ELISA, pg/ml at 1 dpi.

[c] Viremia 2 dpc determined by plaque assay.

[d] Day postchallenge viremia first detected.

[e] Number of digits and snout with vesicles.

[f] Day postchallenge vesicles first detected.

[g] Neutralizing antibody titer at day of challenge.

[h] Neutralizing antibody titer at 14 dpc.

[i] Antibodies (14 dpc) against NS proteins detected by RIP.

antiviral activity and for the level of pIFNα by ELISA as described below.

In experiment 2 nasal swabs were obtained on the day of challenge and daily for 7 dpc. Virus isolation was performed from the swab samples by duplicate inoculation of monolayers of IBRS2 cells in 24-well plates. The monolayers were incubated at 37 °C and 5% CO$_2$ and examined at 24, 48, and 72 h for cytopathic effect. Negative samples were frozen and thawed and a second passage was performed. For positive samples, titration was performed from the original samples by a standard plaque assay in BHK-21 cells.

2.4. IFN assay

Diluted plasma samples, starting at a 1:25 dilution, were placed on IBRS2 cells overnight. Cells were infected with approximately 100 pfu of FMDV serotype A12, overlayed with gum tragacanth, and stained at 24 h with crystal violet [13,18]. Antiviral activity was determined by the highest dilution of plasma that reduced the number of plaques by 50%.

2.5. Porcine IFNα ELISA

A 100 µl aliquot of a monoclonal antibody (MAb) against pIFNα (K9; R&D Systems, Minneapolis, MN) (1 µg/ml) was coated overnight at 4 °C on wells of 96-well plates (Immunolon 2HB; Thermo Labsystems, Franklin, MA) in carbonate–bicarbonate buffer (Sigma, St. Louis, MO). The plates were blocked with PBS containing 0.05% Tween 20 and 5% goat serum (blocking buffer) (Invitrogen, San Diego, CA) for 2 h at 37 °C while shaking and then washed 4× with PBS containing 0.05% Tween 20 (washing buffer) in a Skan Washer 300 (Skatron Instruments, Lier, Norway). One hundred microliter duplicate 10-fold dilutions of plasma samples in minimal essential media (Invitrogen) plus 5% fetal calf serum were added to the plates for 1 h at 37 °C while shaking. The plates were washed 4× as above and 100 µl of a second MAb (F17; R&D Systems) (0.35 µg/ml) was biotinylated following the manufacturers instructions (Pierce Chemical Company, Rockford, IL [29]) and added to the plates for 1 h at 37 °C while shaking. The plates were washed 4× as above and 0.6 µg/ml streptavidin-HRP (KPL, Gaithersburg, MD) in blocking buffer was added and incubated for 30 min at 37 °C while shaking. The plates were washed, the TMB substrate (KPL) added for 15 min, and the reaction stopped by the addition of 100 µl 1 M H$_2$SO$_4$. The plates were read at 450 nm on a Bio-Kinetics Reader EL-312e (Bio-tek Instruments, Winooski, VT). The pIFNα concentrations were determined from a standard curve with recombinant pIFNα (R&D Systems) using Microsoft Excel.

2.6. Radioimmunoprecipitation (RIP) of [^{35}S]-methionine labeled FMDV A24 infected cell lysates

Lysates of FMDV A24 infected IBRS2 cells were incubated with convalescent serum from an FMDV infected

bovine or individual swine sera samples from 0 and 14 dpc and examined for the presence of antibodies specific to FMDV structural and NS polypeptides by RIP [30]. After 60 min incubation at room temperature, antibodies were precipitated with *Streptococcus aureus* protein G. Proteins were resolved by SDS-PAGE on a 15% gel and visualized by autoradiography.

3. Results

We recently demonstrated that swine inoculated with 10^9 pfu Ad5-pIFNα and challenged 1 dpi with virulent FMDV were completely protected from clinical signs of disease and did not develop viremia or antibodies against viral NS proteins including the highly immunogenic NS protein $3D^{pro}$. These animals developed an antiviral response at the earliest time examined, 16 h postinoculation (hpi), and while this response decreased with time it was still detectable for an additional 3–4 dpi [13].

Since animals inoculated with Ad5-pIFNα developed a detectable antiviral response for 4–5 dpi, we were interested in determining how long pIFNα protected these animals against FMDV.

3.1. Duration of protection against FMD by Ad5-pIFNα: clinical and serological response

The control group, challenged 7 days after administration of Ad5-VSVG with virulent, swine-passaged A24 Cruzeiro, did not develop detectable antiviral activity nor was pIFNα detectable by ELISA (Fig. 1, panel A; Table 1). All three animals had viremia at 1 dpc that lasted for 3 additional days and reached levels of 10^5–10^7 pfu/ml (Table 1). All the animals developed fever, had vesicular lesions on the feet at 2 dpc and became severely ill at 3–4 dpc. Two of the animals, 61-6 and 69-8, died at 4 and 7 dpc, respectively. The histopathology findings upon necropsy revealed both animals had necrotic cardiac lesions and death most likely was due to myocardial necrosis as a result of FMDV infection. The surviving animal in this group had a significant FMDV-specific neutralizing antibody response (Table 1).

All animals inoculated with Ad5-pIFNα prior to challenge had high levels of antiviral activity 1 day later, i.e. 200–800 units/ml (Fig. 1, panels B–F). The activity generally declined by the second day, but was detectable for an additional 3–4 days. The levels of pIFNα were directly correlated with the antiviral activity and reached 6–25,000 pg/ml in the plasma. Some of the Ad5-pIFNα inoculated animals developed elevated temperatures, but this only lasted for 1–2 days and the animals were otherwise clinically healthy.

In group 2, inoculated with Ad5-pIFNα and challenged 7 dpi, the last day antiviral activity or pIFNα was detectable was 2–3 days prior to challenge (Fig. 1, panel B). This group developed viremia at 2 dpc, 1 day later than the control group, and the levels were 10–1000-fold lower than the

control group (Table 1). Lesions were first detected 4–5 dpc, 2–3 days later than the control group (Table 1). All animals in the group developed a fever for 1–2 days and had a significant FMDV-specific neutralizing antibody response (Table 1). Animal 70-5 died 11 dpc. The histopathology report upon necropsy found brain stem, thyroid, and lung abscesses and the most likely cause of death was due to the brain abscess and brain swelling probably as a result of bacteremia and was not related to FMD.

All three animals in group 3, challenged 5 dpi, had low but detectable antiviral activity on the day of challenge, but pIFNα was only detectable in one of these animals at this time (Fig. 1, panel C). Only two animals developed viremia, which was first observed at 7 dpc (Table 1), 6 days later than the control group, and was detectable for only 1–2 days. These same animals developed lesions at 8–9 dpc (Table 1) and they were moved to a separate room on the day lesions were observed. The third animal in this group, 70-8, was completely protected from disease even after direct contact for at least 2 days with actively infected animals. All animals developed an FMDV-specific neutralizing antibody response although the titer of the protected animal, 70-8, was significantly lower than the clinically ill animals (Table 1).

The animals in groups 4 and 5, challenged 3 and 1 dpi, respectively, all had both detectable antiviral activity and pIFNα on the day of challenge (Fig. 1, panels D and E). All these animals were completely protected from disease. The animals did not develop viremia (Table 1) or vesicular lesions (Table 1). All the animals had a low, but detectable FMDV-specific neutralizing antibody response after challenge (Table 1).

Two of the three animals challenged 1 day prior to administration of Ad5-pIFNα, group 6, had detectable antiviral activity and pIFNα the day after administration (Fig. 1, panel F). The animal that had the highest level of antiviral activity and pIFNα, 81-6, had the lowest level of viremia, 7×10^1 pfu/ml (Table 1), and never developed vesicular lesions even in the presence of actively infected animals for at least 6 days (Table 1). This animal died 8 dpc. Necropsy revealed that death was probably due to bacterial meningitis as a result of dissemination through the blood from skin abscesses. There was no evidence of FMD infection. The animal that never had detectable antiviral activity or pIFNα, 81-10, had the highest level of viremia, 2.5×10^5 pfu/ml, and had the most severe disease (Fig. 1, panel E; Table 1). All the animals in this group, including the animal that had no clinical signs of disease developed significant FMDV-specific neutralizing antibody responses (Table 1).

3.2. Duration of protection against FMD by Ad5-pIFNα: antibody response against NS proteins

We examined 14 dpc sera by RIP for the presence of antibodies against viral NS proteins as a sensitive assay for measuring challenge virus replication. The surviving animals in groups 1, 2, and 6, all of which developed clinical
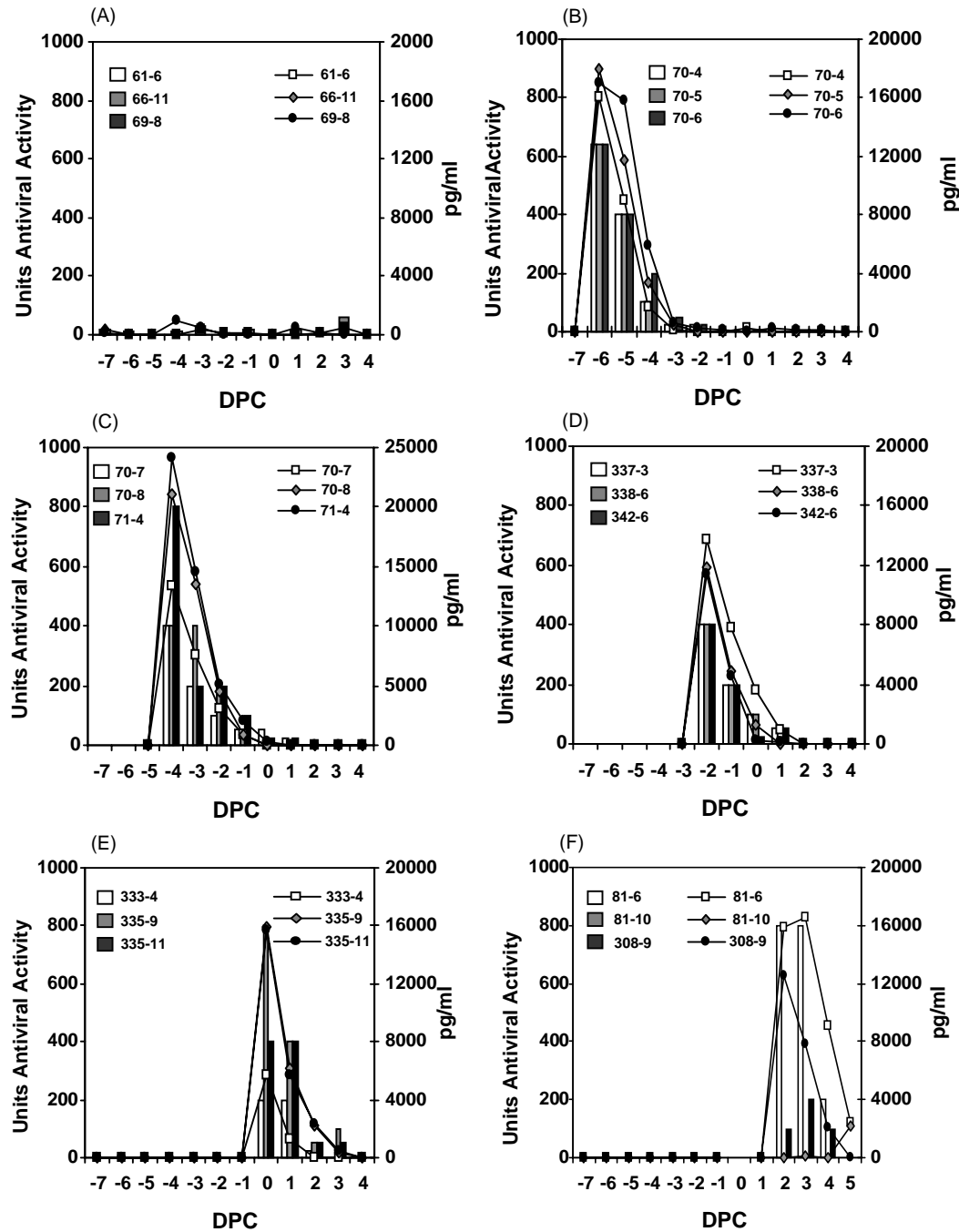
Fig. 1. Interferon response in animals inoculated with Ad5-VSVG or Ad5-pIFNα. Groups of three swine were inoculated IM with 10^9 pfu/animal Ad5-VSVG or Ad5-pIFNα and challenged at various times postinoculation with FMDV A24. Plasma samples were assayed for both antiviral activity by a plaque-reduction neutralization assay and for pIFNα by ELISA. Panel A: inoculated with Ad5-VSVG and challenged 7 dpi with FMDV; panels B–E: inoculated with Ad5-pIFNα and challenged 7, 5, 3, and 1 dpi with FMDV, respectively; panel F: inoculated with Ad5-pIFNα 1 day after challenge with FMDV. The bars represent antiviral activity; the lines represent pg/ml pIFNα.

disease, had antibodies against the viral structural and NS proteins (Fig. 2, lanes 3–5, 9 and 10). The two animals in group 3 that developed clinical disease, 70-7 and 71-4, had antibodies against both viral structural and NS proteins (lanes 6 and 8), while 70-8, an animal that did not develop viremia or clinical disease, only had antibodies against viral structural proteins (lane 7). This animal had a low, but detectable FMDV-specific neutralizing antibody response at 14 dpc. The animals in groups 4 and 5, none of which had clinical or serological signs of infection, had only low levels of antibodies against the viral structural proteins and no detectable antibodies against viral NS proteins (lanes 11–16).

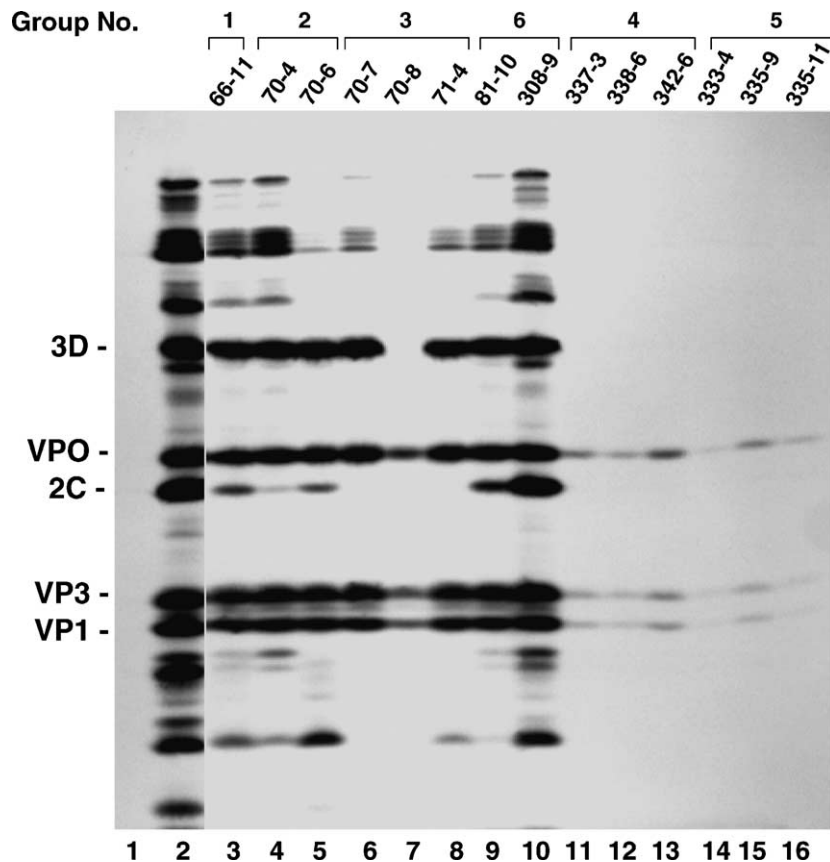We also used a 3ABC ELISA as an additional method to serologically distinguish Ad5-pIFNα inoculated protected

Fig. 2. Examination by RIP of 14 dpc serum from inoculated animals for antibody response against FMDV structural and NS proteins. [^{35}S]-Methionine labeled cell lysates from FMDV A24 infected IBRS2 cells were immunoprecipitated with various sera. Lane 1: 0 dpc serum from swine 61-6; lane 2: convalescent serum from an FMDV infected bovine; lanes 3–16: 14 dpc serum from the indicated animals. Immunoprecipitated samples were examined by SDS-PAGE on a 15% gel.

animals from control, unprotected animals. The results obtained were identical to the RIP data except for animal 70-8 (data not shown). This animal never developed disease, had a low but detectable FMDV-specific neutralizing antibody response and had no antibodies against the viral NS proteins by RIP yet was positive in a 3ABC ELISA.

3.3. Co-administration of Ad5-pIFNα and Ad5-A24: antiviral response prechallenge

Since animals challenged 1 or 3 days after Ad5-pIFNα inoculation were completely protected from disease, while the animals challenged 5 dpi were either protected or had delayed and less severe clinical disease, we wanted to determine if dual administration of an antiviral and a vaccine could prolong protection.

We have previously demonstrated that animals inoculated once with Ad5-A24 were completely protected when challenged 7 days later with A24 [7]. In this experiment, groups of animals (three per group) were inoculated with Ad5-VSVG (group 1, control), Ad5-A24 (group 2), Ad5-pIFNα (group 3), or Ad5-pIFNα and Ad5-A24 (group 4) (Table 2). Animals were challenged 5 days later with virulent, swine-passaged A24.

All inoculated animals, including the animals in groups 1 and 2, developed an antiviral response and detectable pIFNα by 4 or 10 hpi (Fig. 3, panels A–D). However, by 1 dpi the animals in groups 1 and 2 had little or no detectable antiviral activity or pIFNα, while the animals inoculated with Ad5-pIFNα, groups 3 and 4, had increased levels of antiviral activity and pIFNα. On the day of challenge only the animals in groups 3 and 4 had low but detectable antiviral activity, except for 9974 (group 4). Surprisingly in the group inoculated with Ad5-pIFNα and Ad5-A24 the level of pIFNα decreased more rapidly than the group inoculated with Ad5-pIFNα alone.

3.4. Co-administration of Ad5-pIFNα and Ad5-A24: clinical and serological response after challenge

The results of clinical and serological responses in all groups are summarized in Table 2. All animals in the control group developed viremia by 1 dpi, which lasted for 2–3 additional days (Table 2). These animals also had low levels of virus in nasal swabs at 1 dpc, i.e. 10^1–10^2 pfu/ml, that lasted for 2–3 days. The animals had lesions at 2–3 dpc that became severe 3–4 dpc (Table 2; data not shown) and all animals seroconverted by 4 dpc (data not shown).
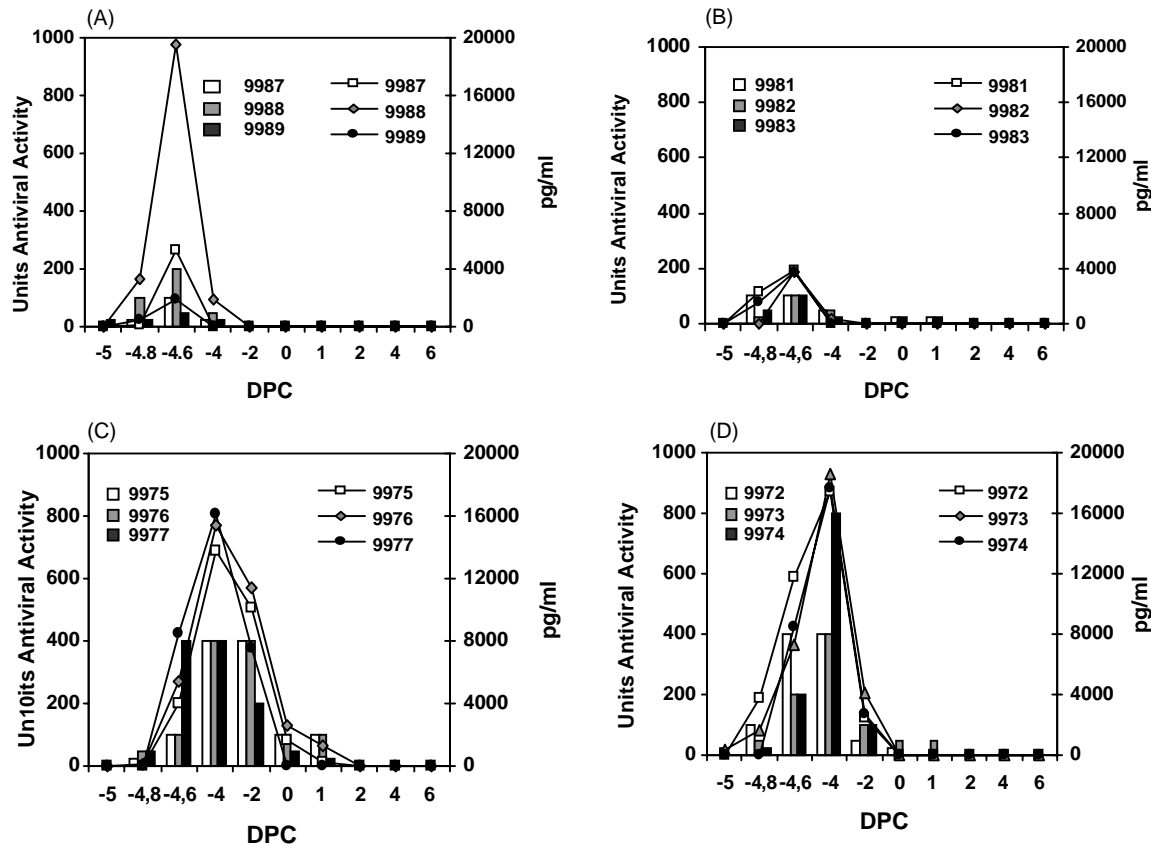
Fig. 3. Interferon response in animals inoculated with Ad5-VSVG, Ad5-A24, Ad5-pIFNα or a combination of Ad5-A24 and Ad5-pIFNα. Groups of three swine were inoculated IM with Ad5-VSVG, Ad5-A24, Ad5-pIFNα or a combination of Ad5-A24 and Ad5-pIFNα and challenged 5 dpi with FMDV A24. Plasma samples were assayed for both antiviral activity by a plaque-reduction neutralization assay and for pIFNα by ELISA. Panel A: inoculated with Ad5-VSVG; panel B: inoculated with Ad5-A24; panel C: inoculated with Ad5-pIFNα; panel D: inoculated with a combination of Ad5-A24 and Ad5-pIFNα. The bars represent antiviral activity; the lines represent pg/ml pIFNα.

In group 2, inoculated with Ad5-A24, only 1 animal had a low level of viremia, 10^2 pfu/ml, for 1 day (Table 2), but all animals had virus in nasal swabs, 10^1–10^2 pfu/ml, for 1–2 days. The animal's only developed lesions 3 dpc, 1 day later than the control group and the severity of disease was significantly reduced compared to the control animals (Table 2). The two animals that did not have detectable viremia, 9981 and 9982, developed 2 and 6 lesions, respectively. These animals had a low, but detectable, FMDV-specific neutralizing antibody response on the day of challenge and the titers were significantly boosted 4 dpc (Table 2; data not shown).

All of the animals in groups 3 and 4 inoculated with Ad5-pIFNα or Ad5-pIFNα and Ad5-A24, respectively, were completely protected from disease (Table 2). The animals did not develop viremia, virus in nasal swabs, fever, or vesicular lesions after challenge. The animals inoculated with only Ad5-pIFNα had a low FMDV-specific neutralizing antibody response by 14 dpc (Table 2). Group 4 animals inoculated with both Ad5-pIFNα and Ad5-A24 had a low neutralizing antibody response on the day of challenge (essentially identical to group 2) and their titer was significantly boosted by 9 dpc to PRN_{70} 320–800.

3.5. Co-administration of Ad5-pIFNα and Ad5-A24: antibody response against NS proteins

The animals in groups 1 and 2, all of which developed clinical disease, had antibodies against the viral structural and NS proteins (Fig. 4, lanes 9–14). In contrast, the animals in groups 3 and 4, which were completely protected from clinical disease and viremia, only developed antibodies against the viral structural proteins and did not have antibodies against the viral NS proteins using either 14 dpc serum (lanes 3–8), or 21 dpc serum (data not shown), suggesting the induction of sterile immunity. Examination of the serological response, at 14 and 21 dpc, by a 3ABC ELISA demonstrated the absence of 3ABC antibodies in all animals in groups 3 and 4 and a positive response in all animals in groups 1 and 2 (data not shown). Interestingly, the animals in group 4, inoculated with the combination antiviral and vaccine formulation, had both higher FMDV-specific neutralizing antibody titers and higher antibody titers against the viral structural proteins than the group inoculated only with the antiviral (compare Table 2 and Fig. 4, lanes 3–8). Thus, exposure of dual-inoculated animals to virus enhanced
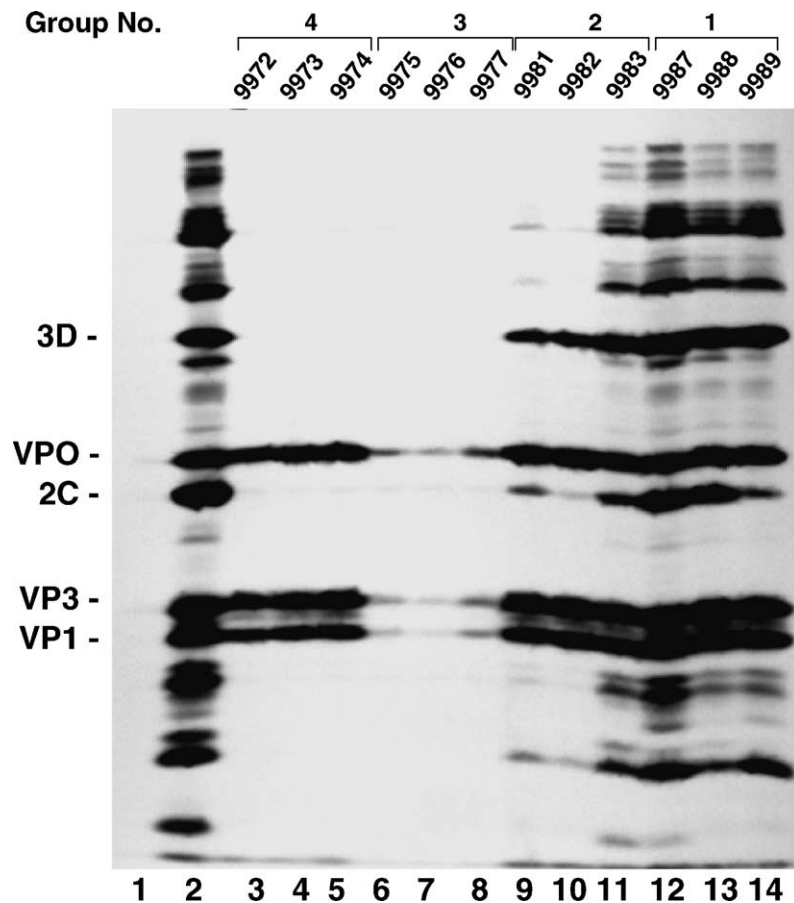
Fig. 4. Examination by RIP of 14 dpc serum from inoculated animals for antibody response against FMDV structural and NS proteins. [^{35}S]-Methionine labeled cell lysates from FMDV A24 infected IBRS2 cells were immunoprecipitated with various sera. Lane 1: 0 dpc serum from swine 9989; lane 2: convalescent serum from an FMDV infected bovine; lanes 3–14: 14 dpc serum from the indicated animals. Immunoprecipitated samples were examined by SDS-PAGE on a 15% gel.

the level of the antigen specific immune response without resulting in disease or infection.

4. Discussion

The recent outbreaks of FMD in a number of previously disease-free countries have demonstrated the severe economic consequences that can occur if FMD is not rapidly detected and controlled [4,5]. The control strategies utilized in the UK in 2001 included culling of infected and in-contact susceptible animals, but not vaccination. This strategy allowed the UK to resume FMD-free status and trade with disease-free countries 3 months after the absence of disease had been certified. In the 2001 FMD outbreak in The Netherlands culling as well as ring vaccination and slaughter of all vaccinated animals were the approaches used and The Netherlands was also able to resume trade 3 months later. However, the slaughter of millions of animals in the UK, many of which may have been disease-free, and the difficulty of disposing of so many carcasses raised the concern of many segments of society.

Following the FMD outbreak, the UK commissioned a number of inquiries to examine the performance of the government in dealing with the situation and to recommend a strategy in the event of subsequent outbreaks [31]. One of the main recommendations was to include vaccination as a part of future control of an FMD outbreak. The inquiry of "infectious diseases in livestock" by the Royal Society recommended emergency vaccination as a "major tool of first resort" to prevent an FMD outbreak becoming an epidemic and added that the aim is "vaccination-to-live" [32]. This later report also recommended development of both improved vaccines and modern diagnostic methods.

We have proposed a combination FMD control strategy that will induce both rapid and long-term protection and in addition, eliminate many of the drawbacks of the current vaccine. We have identified a mechanism of rapidly protecting animals from FMD by delivery of pIFNα via a replication-defective adenovirus. Swine are protected from FMDV challenge 1 day after administration of Ad5-pIFNα and probably within 10–16 h [13]. Our results in this study indicate that IFNα alone can protect swine for at least 3–5 dpi. The duration of protection is dependent on the vector

dose and subject to animal variability including the physical health of the animals. For instance, we believe complete protection of only one of three animals challenged 5 days after Ad5-pIFNα administration in the first experiment, while all three animals given this vector in the second experiment were protected, can be attributed to the presence of a pre-existing skin rash on all the animals in the first experiment that in some cases may have compromised their health.

The use of vaccination to induce relatively long-term protection is a second component of our disease control strategy. We have developed a multiepitope immunogen, empty viral capsids, delivered via a replication-defective adenovirus that can protect swine from virus challenge as early as 7 days after a single vaccination [7].

It was anticipated that the immediate protection afforded by an antiviral would allow development of a long-term protective adaptive immune response induced by co-administration of a vaccine. In this study, we demonstrated that vaccination alone begins to induce a protective response by 5 dpi, but this approach is not sufficient to completely protect animals. The combination of Ad5-pIFNα and Ad5-A24 induced sterile immunity when the animals were challenged 5 days later. However, since in this latter experiment, administration of Ad5-pIFNα alone protected all animals challenged 5 days later, it is not possible to characterize the efficacy of the combination approach in early protection. Nevertheless, based on the significant boost in the FMDV-specific neutralizing antibody response of the dual-inoculated group after challenge as compared to the Ad5-pIFNα inoculated group it would appear that the combination approach does afford animals exposed to FMDV early after treatment both rapid protection and a more robust neutralizing antibody and presumably protective response than either approach alone.

Studies indicate that IFNα/β, in addition to directly inducing an antiviral response has an immunomodulatory role [33], and can enhance the humoral immune response to soluble antigens [34] as well as act as an adjuvant when administered with a human influenza subunit vaccine [35]. Thus, the co-administration of Ad5-pIFNα may offer additional advantages to our combination strategy by enhancing the vaccine-induced adaptive immune response. Experiments are planned to directly examine the ability of pIFNα as well as pIFNβ to act as an adjuvant for our Ad5-FMDV empty viral capsid vaccine.

It was interesting to note that animals challenged 7 days after Ad5-pIFNα administration had no detectable IFNα in their plasma for 2–3 days prior to challenge; yet they had delayed and lower levels of viremia and vesicular lesions as compared to the control animals. This data implies that although complete resistance to virus replication may only last 1–2 days after clearance of IFNα, IFN "activated" cells can still reduce the rate of virus replication for an additional 1–2 days. We have previously demonstrated that in cell culture double-stranded RNA-dependent protein kinase (PKR) and RNase L are IFN-induced genes that play a role in inhi-

bition of FMDV replication [18]. It would be of interest to directly demonstrate that these proteins are induced in IFN treated animals, determine both the half-life of the above proteins and their mRNAs, as well as identify other potential antiviral molecules induced in activated cells from IFNα treated animals.

The addition of pIFNα 1 dpc still reduced the level of viremia and severity of disease and in one animal prevented clinical disease. This suggests that antiviral treatment can be used as a therapeutic to reduce both disease severity and the amount of virus shed by infected animals as well as a prophylactic to inhibit virus replication in disease-free animals. The two animals in this group that had detectable antiviral activity and pIFNα had reduced levels of viremia and no or delayed appearance of vesicular lesions. In fact, the animal that had the highest antiviral activity and pIFNα levels, 81-6, had the lowest levels of viremia, 7×10^1 pfu/ml, and never developed vesicular lesions. In contrast the animal that had no detectable antiviral activity or pIFNα, 81-10, had as severe disease as the control animals. It is not clear why this animal had no detectable IFNα, since we have administered this dose of Ad5-pIFNα to more than 24 animals and each of these animals developed detectable antiviral activity in their plasma.

A number of groups have shown that treatment with IFNα/β can block or reduce virus replication in various animal models or in naturally susceptible animals [35–41]. IFNα is also used therapeutically in individuals with chronic hepatitis C [42,43] or hepatitis B [44,45] and a combination of pegylated IFNα and ribavirin has eliminated detectable viral RNA in at least 40% of hepatitis C treated individuals [43]. These results, as well as our data, suggest that administration of IFNα may be a useful therapeutic approach to eliminate the FMDV carrier state.

Our results indicate the potential of a combination antiviral and vaccine approach as a FMD control strategy in the event of an outbreak in disease-free countries. Susceptible animals that are outside of an infected zone could be administered both vectors and be sterilely protected from disease. If a policy of vaccination-to-live was in practice, these animals could easily be distinguished from infected animals using a 3D ELISA. Within the zone encompassing the outbreak, infected animals would still have to be destroyed, but early administration of Ad5-pIFNα could at least reduce the level of virus replication and shedding, thereby effectively decreasing the spread of virus. We believe that this approach could significantly reduce the number of animals that would have to be destroyed in a disease incursion, thereby ameliorating the environmental and social impact of an FMD outbreak.

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